THE ENZYMATIC HYDROXYLATION OF TRYPTOPHAN

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Received April 28, 1961

Although much is known about the role and metabolism of 5-hydroxyindole (5-HI) compounds the initial hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP) has not been shown to occur in vitro (Symposium, 1959, Udenfriend, et al., 1960) in animal tissues despite previous attempts by several workers (Dalgliesh and Dutton, 1957, Ichihara, et al., 1957) to demonstrate this conversion. A possible relationship between disturbances in phenylalanine and tryptophan metabolism (Pare, et al., 1958, Baldridge, et al., 1959) caused us to renew the search for a tryptophan hydroxylating system (THS). Since previous results showed that excess dietary tryptophan increased one of these enzymes, phenylalanine hydroxylase, in the liver (Freedland, et al., 1960) it was thought that this would be the organ of choice to look for a THS.

The following methods were used for the assay of THS. Male albino rats weighing between 200 and 300 grams were sacrificed and the livers rapidly removed and a portion homogenized in a Potter-Elvehjem homogenizer for one minute in two volumes of chilled isotonic KCl + 0.00025 M NaOH then centrifuged at 20,000 x g for 45 minutes at 0-4°C. The resulting supernatant solution was used as the source of THS. Each assay determination contained 0.2 ml of 1.0 M phosphate buffer pH 6.7; 0.2 ml of DPN (56 mg/20 ml); 0.2 ml of nicotinamide (120 mg/20 ml); enzyme source (0.0 - 1.0 ml); and substrate (0.4 ml of 0.1 M L-tryptophan) or substrate plus inhibitor; the final volume

was made to 2.5 ml with water.

The reaction was carried out for one hour (except for the time studies) at 38°C. with constant shaking, stopped with 1.0 ml of 20% trichloroacetic acid, centrifuged and the supernate assayed for hydroxyindole (HI) compounds by a modification of the 1-nitroso-2-napthol reaction method Udenfriend et al., (1955, a). Since it has been shown that tryptamine is not hydroxylated by the rat to form either hydroxytryptamine or hydroxyindole acetic acid (Udenfriend, et al., 1959) it has been assumed that an increase in HI concentration indicates hydroxylation of tryptophan regardless whether the product was hydroxytryptophan, hydroxytryptamine, or hydroxyindole acetic acid.

Increasing amounts of HI are formed with longer periods of incubation (Table 1) but the rate of formation decreases with time; also increasing amounts of the enzyme solution produced increasing amounts of HI although not in a linear fashion. The reason for this non-linearity with concentration is not clear at present, but is probably not due to a simple activator or inhibitor. The ability of this system to hydroxylate tryptophan is markedly decreased by boiling, adding strong base or acid, or omitting the DPN or sufficient O_2. These facts further indicate that this hydroxylation is enzymatic and not a non-specific chemical hydroxylation.

Next an attempt was made to identify the product of this hydroxylation. When the reaction was stopped by the addition of 0.85 ml of 12 N HCl and the precipitate removed, the resulting solution showed a strong 550 mu emission peak when activated by 295 mu light. When tryptophan was added at the end of the reaction as a control no 550 mu peak was observed. The 550 mu peak is a property of 5-hydroxyindole compounds (Bogdanski, et al., 1956). When the assay mixture was adjusted to pH 3, saturated with NaCl and extracted with ether all of the hydroxylated product remained in the aqueous layer indicating that the product was not 5-hydroxyindole acetic acid (5-HIAA) (Udenfriend, et al., 1955, b). After a readjustment of the pH to 10 practically all of the hydroxylated product could be extracted into n-butanol, a property similar to that of

TABLE 1

EFFECT OF TIME, ENZYME CONCENTRATION, AND OTHER FACTORS ON THE FORMATION OF HYDROXYINDOLES

Volume of Supernate (ml)	Time (min)	Modification of assay	Mu moles of hydroxy-indole formed/2.5 ml incubation mixture*
1.0	15		78
1.0	30		127
1.0	60		201
1.0	120		231
0.25	60		39
0.50	60		127
0.75	60		167
1.0	60	0.2 ml 1 N HCl	<20
1.0	60	0.2 ml 1 N NaOH	<20
1.0	60	Boiled first	< 5
1.0	60	- DPN	29
1.0	60	Anaerobic	<10

*All values are corrected by substracting a similar incubation which has the tryptophan added after the addition of trichloroacetic acid. Tryptophan blank was equivalent to less than 5 mu moles of hydroxyindole.

5-hydroxytryptamine (5-HT) (Udenfriend, et al., 1955, b). The butanol extracted reaction product was then re-extracted into 0.1N HCl, the nitrosonaphthol reaction performed and the spectrum of the resulting chromophore was identical to that obtained with commercial serotonin. This semi-purified product also exhibited a fluorescence spectra in 3 N HCl identical to that of serotonin. A biological assay, using guinea-pig ileum, of the extracted product indicated the presence of serotonin corresponding to 84-105 per cent (99 $\pm$ 3% for six individual determinations) of that calculated from results of the nitroso-naphthol reaction. The extracted product was then chromatographed in three different solvents (Armstrong, et al., 1958). The following R_f values were obtained for 5-HT, tryptophan, and the enzymatically formed product respectively: aqueous KCl 20% - .35, .53, .35; butyl alcohol, acetic acid and water (8:1:1) - .41, .49, .41; isopropylalcohol, ammonium hydroxyide, water (8:1:1) - .56, .35, .55. Thus, the enzymatically formed product also has

chromatographic properties similar to 5-HT in at least three different solvents.

When the extracted reaction product was neutralized and added to crude whole liver homogenate it could be completely converted to a product which could be extracted like 5-HIAA (Udenfriend, et al., 1955, b), indicating the serotonin fraction is biologically capable of being converted to the next suspected compound on its metabolic pathway. We have also obtained evidence that a substance with the properties of 5-hydroxytryptophan accumulates when appropriate decarboxylase inhibitors are added to the incubation mixture. Thus, it appears that the liver contains an enzyme system capable of hydroxylating L-tryptophan to form physiologically active 5-hydroxytryptophan derivatives and with the described systems all the intermediate products along this pathway can be obtained. It was also noted in our experiments that tryptamine was not converted to 5-HT under conditions which did convert tryptophan.

This work was supported by grants A-4732 and M-2730 provided by the National Institutes of Health.

ACKNOWLEDGEMENTS

Dr. Erminio Costa, Thudicum Psychiatric Research Laboratory, Galesburg State Research Hospital, Galesburg, Illinois, kindly performed the bioassays for 5-hydroxytryptamine. Dr. Motosuke Kaihara and Dr. Raymond Brown, Cancer Research Hospital, University of Wisconsin Medical School aided greatly in the spectrophotofluorometric determinations.

REFERENCES

Armstrong, M. D., Shaw, N. F. K., Gortatowski, M. J., and Singer, H., J. Biol. Chem. 232, 17 (1958).

Baldridge, R. C., Bowfsky, L., Baird, H., III, Reichle, F., and Bullock, D., Proc. Soc. Exp'l. Biol. Med., 100, 529 (1959).

Bogdanski, D. F., Pletscher, A., Brodie, B. B., and Udenfriend, S., J. Pharmacol. and Exp. Therap. 117, 82, (1956).

Dalgliesh, C. E. and Dutton, R. W., Brit. J. Cancer, X1, 296 (1957).

Freedland, R. A., Krakowski, M.C., and Waisman, H.A., Federation Proc. 19, 7, (1960).

Ichihara, K., Sakamoto, H., Inamori, K., and Sakamoto, Y., J. Biochem. (Japan) 44, 649 (1957).

Pare, C. M. D., Sandler, M., and Stacey, R. S., Lancet, II, 1099 (1958).

Symposium on Tryptophan Metabolism, 136th A.C.S. Meeting, Atlantic City, Sept. 14, 1959, p. 13.

Udenfriend, S., Weissbach, H., and Clark, C.T., J. Biol. Chem., 215, 337 (1955, a).

Udenfriend, S., Titus, E., and Weissbach, H., J. Biol. Chem. 216, 499 (1955, B).

Udenfriend, S., Creveling, C. R., Posner, H., Redfield, B. G., Daly, J., and Withop, B., Arch. Biochem. Biophys. 83, 501 (1959).

Udenfriend, S., Weissbach, H., and Mitoma, C., Annual Reviews of Biochemistry, 29, 207 (1960).